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Characterization of a further micro-immunofluorescence serotype of *Chlamydia:* TRIC Type G

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The micro-immunofluorescence (micro-IF) test was developed by Wang and Grayston (1970); their results and the practicability of the test were confirmed by Treharne, Katzenelson, Davey, and Gray (1971) and Treharne, Davey, Gray, and Jones (1972). The test has clearly established the existence of six TRIC (trachoma-inclusion conjunctivitis) serotypes that correspond with six types defined by mouse toxicity prevention tests (Alexander, Wang, and Grayston, 1967) and three LGV (lymphogranuloma venereum) serotypes that correspond with three types defined by plaque-reduction neutralization tests (Schachter, 1971).

More than 100 iodine-positive *Chlamydia* Subgroup A (Gordon and Quan, 1965) isolates have been serotyped in our laboratory using the micro-IF test. One isolate (TRIC/G/GB/IOL-238/R) secured from the rectum of a patient (Dunlop, Hare, Darougar, and Dwyer, 1973) has consistently failed to fit Wang and Grayston's existing classification of serotypes (Treharne and others, 1971; Treharne and others 1972). A further six isolates have been shown to be immunologically identical to IOL-238 in the micro-IF test and thus form a new serotype. This paper records the immunological and biological characteristics of the new serotype, which lead to the proposal to designate it as TRIC Type G, since it lies between TRIC Type F and LGV III serotypes.

Methods

GROWTH IN THE YOLK SAC OF THE DEVELOPING CHICK EMBRYO

Yolk sac titrations were carried out using 6-day-old fertile hens' eggs. ELD_{50} and occasionally EID_{50} determinations were made, using the formula of Reed and Muench (1938). Total particle counts of suspensions were made by the method of Reeve and Taverne (1962).

SULPHADIAZINE SENSITIVITY

From preliminary yolk sac titrations, the ELD_{50} of each isolate that would kill the inoculated chick embryos in 9 to 10 days was calculated. Twenty to thirty developing chick embryos inoculated by the yolk sac route were used for the sensitivity determination of each isolate. Half of the embryos in each group were inoculated additionally with 0·1 ml. sterile distilled water containing 1 mg. sulphadiazine; the remaining half (control group) received 0·1 ml. sterile distilled water without sulphadiazine. The eggs were incubated for a maximum of 14 days and the difference between the average day of death (ADD) of the sulpha-treated and untreated embryos was calculated for each isolate.

PRESENCE OF GLYCOGEN IN INCLUSIONS IN CELL CULTURE

Four irradiated McCoy cell monolayers were inoculated with 5 per cent. (w/v) yolk sac suspensions of each isolate and centrifuged at 2,700 G. for 60 min. at 35°C. Tubes were incubated at 35°C. for 24 hrs and the medium was then changed. After a further 48 hrs' incubation at 35°C., the coverslips bearing the cell monolayers were removed from individual tubes and two coverslips for each isolate were stained with an iodine stain (Gordon and Quan, 1965) for microscopy to determine the presence of glycogen. The other two coverslips of each isolate inoculum were stained with Giemsa stain.

The Subgroup B organism, meningopneumonitis (Cal-10) strain, was included as a negative control.

PATHOGENICITY FOR MICE

Each of the seven Type G isolates, the LGV Type III isolate IOL-253, and the meningopneumonitis agent Cal-10 were titrated in three-week-old white mice by the intracerebral route. Ten-fold dilutions of 50 per cent. (w/v) yolk sac suspensions of every

isolate were prepared, and 0·03 ml. of each dilution of each isolate was inoculated into the brain of each mouse. (Ten mice were used for each dilution.) Control mice were inoculated with 10-fold dilutions of normal non-infected yolk sac suspension. All animals were observed daily for 4 weeks and signs of paralysis and death were recorded.

PATHOGENICITY FOR BABOONS

Two 10-15 kg. baboons (*Papio cynocephalus*), one male and one female, were inoculated with isolate IOL-238/R by a variety of routes. In both animals conjunctiva, rectum, and knee joint were inoculated and additionally the urethra in the male and cervix in the female. Clinical examinations, with collection of specimens for microscopy and chlamydial isolation in cell culture, were made regularly of both animals.

MICRO-IF SEROTYPING TEST

All isolations made in our laboratory (IOL) listed in Table I were originally isolated in irradiated McCoy cell cultures. Antigen production for the micro-IF test was carried out by inoculating heavily infected cell culture harvest into the yolk sacs of developing chick embryos. Only heavily infected yolk sacs from surviving embryos were used for the test. 5 per cent. (w/v) suspensions of infected yolk sac were used as slide antigens and 1 per cent. (w/v) suspensions were used for the production of hyperimmune sera in mice. All other methods used in the micro-IF test were similar to those reported previously by Wang and Grayston (1970) and Treharne and others (1971). Results were analysed and additional specificity differences (SPD) were calculated according to the formula of Fraser and Berman (1965).

TABLE I *Isolates designated as* TRIC *micro-IF serotype G*

Patients case code	*Full designation of isolate*	*Site of origin*	*Abbreviated designation*
Mr. JD	TRIC/G/GB/IOL-200/GU	Urethra	IOL-200
Mrs. JD	TRIC/G/GB/IOL-201/GCx	Cervix	IOL-201
Mrs. JD	TRIC/G/GB/IOL-238/R	Rectum	IOL-238
Baby JD	TRIC/G/GB/IOL-202/ON	Conjunctiva	IOL-202
Mrs. JO (i)	TRIC/G/GB/IOL-241/GCx	Cervix	IOL-241
Mr. JO	TRIC/G/GB/IOL-215/GU	Urethra	IOL-215
*392/OC[a]	TRIC/G/USA-Cal/Cal-392/OC	Conjunctiva	Cal-392

[a]Isolated by Dr. J. Schachter (San Francisco, USA).

Results

GROWTH IN YOLK SAC

The seven Type G isolates tested were only moderately lethal for the chick embryo with a $\log_{10}$ ELD_{50}/ml. ranging from 1·8 to 3·8, whereas the LGV agent had a titre of 8·8 $\log_{10}$ ELD_{50}/ml. Infectious titres for eggs were generally 1·0 to 1·5 logs higher than the lethal titres (Table II).

The ELD_{50}/total particle ratio measured for one of the Type G isolates (IOL-238) was 1:9000, whilst for LGV agent IOL-253 it was 1:120 and for the meningopneumonitis agent 1:18.

TABLE II *Biological characteristics of isolates*

Isolate no.	*$Log_{10}ELD50$/ml.*	*Type of inclusion*	*Glycogen in inclusion*	*Sulphadiazine sensitive*	*Mouse pathogenicity*	
					Intracerebral	*Intravenous*
IOL-200	3·8 (5·2)[a]	Compact	Yes	Yes	—	—
IOL-201	2·3	Compact	Yes	Yes	—	—
IOL-202	2·4	Compact	Yes	Yes	—	—
IOL-215	1·8	Compact	Yes	Yes	—	—
IOL-238	2·1 (3·5)	Compact	Yes	Yes	—	—
IOL-241	3·2 (4·8)	Compact	Yes	Yes	—	—
Cal-392	1·8	Compact	Yes	Yes	—	—
LGV/IOL-253	5·5 (6·2)	Compact	Yes	Yes	(2·3)[b]	—
Meningopneumonitis	8·8	Diffuse	No	No	(6·5)	+

[a]Figures in parenthesis are $\log_{10}$EID50/ml. titres.
[b]Figures represent $\log_{10}$MLD50/ml. titre.

GROWTH IN IRRADIATED McCOY CELLS

All Type G isolates produced only very minor degenerative cytopathic effects in irradiated McCoy cells. Compact inclusions were formed in vacuolated areas of the cytoplasm and these inclusions were shown to contain glycogen when stained with an iodine solution, 48 hrs after inoculation. The LGV agent IOL-253 gave similar results. On the other hand, the Subgroup B agent meningopneumonitis produced a rapid degeneration of the cells accompanied by occasional cells showing diffusely spread inclusions which failed to stain with iodine (Table II).

TABLE IIIA *Mean percentage cross-reactions between* TRIC *and LGV serotypes*

Mouse antisera	*Slide antigens*												
	C	*A*	*B*	*Ba*	*E*	*D*	*F*	*G*	*LIII*	*LII*	*LI*	*'UW'-4'*	*'UW'-12'*
C	0	5	13	12	12	12	14	13	14	13	13	14	9
A	5	0	14	12	13	13	14	13	14	14	14	13	11
B	13	14	0	0	8	9	14	12	12	12	12	13	14
Ba	12	12	0	0	3	3	10	7	6	3	3	12	13
E	12	13	8	3	0	2	10	7	5	5	5	13	13
D	12	13	9	3	2	0	9	5	5	5	5	13	13
F	14	13	14	10	10	10	0	3	9	11	12	13	13
G	13	13	12	7	7	7	3	0	4	6	6	12	12
LIII	14	14	12	6	5	5	9	4	0	6	5	7	13
LII	13	14	12	3	5	5	11	6	6	0	3	10	14
LI	13	14	12	3	5	5	12	6	5	3	0	11	14
UW-4[a]	14	13	13	12	13	13	13	12	7	10	11	0	12
*UW-12[a]	9	11	14	13	13	13	13	12	13	14	14	12	0

[a]Two undefined serotypes supplied by Dr. S. P. Wang, Seattle

TABLE IIIB *Mean specificity differences (SPD) between* TRIC *and LGV serotypes*

Mouse antisera	*Slide antigens*												
	C	*A*	*B*	*Ba*	*E*	*D*	*F*	*G*	*LIII*	*LII*	*LI*	*UW-4*	*UW-12*
C	100	25											6
A	13	100											
B			100	100	13	6							
Ba			100	100	50	50	13	13	100	100	100		
E				25	100	100	6	13	25	25	50		
D				25	25	100	13	13	25	25	50		
F							100	25					
G				6	6	6	50	100	50	25	13		
LIII					13	13	6	13	100	13	13		
LII				13	13	13		13	13	100	25		
LI				13	6	6		13	25	50	100		
UW-4									13	6		100	
UW-12													100

No score = per cent. cross-reaction less than 6

SULPHADIAZINE SENSITIVITY

The lethal effect on chick embryos of all Type G isolates and the LGV agent IOL-253 was shown to be inhibited by the addition of 1 mg. sulphadiazine; meningopneumonitis agent was not, however, inhibited by this concentration of the drug (Table II).

PATHOGENICITY FOR MICE

No paralysis or deaths occurred in any of the mice inoculated intracerebrally with Type G isolates. Titration of the LGV isolate IOL-253, however, caused some early deaths in mice between 24 and 48 hrs, with a few mice dying between the 3rd and 10th days, the resulting MLD_{50} being $\log_{10} 2{\cdot}3$ (Table II). In contrast, the meningopneumonitis agent Cal-10 was very lethal with an MLD_{50} of $\log_{10} 6{\cdot}3$.

PATHOGENICITY FOR BABOONS

The experimental inoculation of baboons with TRIC Type G has been reported elsewhere (Darougar, Kinnison, and Jones, 1971); suffice it to say here that a mild follicular conjunctivitis accompanied by exudate and hyperaemia developed in both animals. Inclusions were detected by microscopy in smears of conjunctival scrapings and *Chlamydia* was isolated in irradiated McCoy cell cultures up to 2 weeks after inoculation in the male and 7 weeks in the female.

A mild urethritis was shown histologically in the male baboon at necropsy and chlamydial agent was isolated in cell culture from the urethra for 2 weeks after the inoculation. No evidence of cervical infection could be detected in the female baboon.

Slight clinical reactions developed in the knee joints of both animals but *Chlamydia* could not be detected in aspirated material.

No clinical or microbiological evidence of rectal infection by *Chlamydia* could be found.

MICRO-IF SEROTYPING

In numerous repeat micro-IF two-way cross-reaction tests, all the Type G isolates were shown to be consistently different from any of the other known TRIC or LGV serotypes (Table IIIA and B, p. 297).

All Type G isolates, however, behaved in a closely similar manner, there being only minor specificity differences (Fraser and Berman, 1965) that lie within the range of experimental error (Table IVA and B).

Table IIIB shows the interrelations between serotypes expressed as percentage cross-reactions (Wang and Grayston, 1970).

TABLE IVA *Series of repeat micro-IF cross-tests showing specificity differences (SPD) between* TRIC *Type G isolates*

Mouse antisera	*Test no.*	*Slide antigens* IOL-238	IOL-200	IOL-241	Cal-392
IOL-238	1	0 (128)[a]	−1 (256)	0 (256)	0 (128)
	2	0 (128)	−1 (256)	0 (256)	0 (128)
	3	0 (128)	0 (128)	0 (128)	0 (128)
IOL-200	1	−1 (128)	0 (128)	1 (128)	0 (128)
	2	−1 (256)	0 (256)	1 (256)	0 (256)
	3	0 (256)	0 (256)	1 (256)	0 (256)
IOL-241	1	0 (64)	1 (64)	0 (128)	1 (64)
	2	0 (64)	1 (64)	0 (128)	1 (128)
	3	−1 (256)	−1 (256)	0 (128)	1 (64)
Cal-392	1	0 (256)	0 (256)	1 (256)	0 (256)
	2	0 (256)	0 (256)	1 (128)	0 (256)
	3	0 (256)	0 (256)	1 (256)	0 (256)

[a] ( ) Figures in parenthesis represent reciprocal cross-reaction titres

Discussion

The results of micro-IF typing of isolates in our laboratory have indicated the existence of six that are different from all the established TRIC and LGV serotypes (Treharne and others, 1972). The specificity differences (SPD) between these isolates which are to be designated as TRIC Type G and the further

TABLE IVB *Series of repeat micro-IF cross-tests showing percentage cross-reactions between* TRIC *Type G isolates*

Mouse antisera	*Test no.*	*Slide antigens* IOL-238	IOL-200	IOL-241	Cal-392
IOL-238	1	100	200	200	100
	2	100	200	200	100
	3	100	100	100	100
IOL-200	1	100	100	100	100
	2	100	100	100	100
	3	100	100	100	100
IOL-241	1	50	50	100	50
	2	50	50	100	100
	3	200	200	100	50
Cal-392	1	100	100	100	100
	2	100	100	50	100
	3	100	100	100	100

isolate 392/OC kindly supplied by Dr. J. Schachter from San Francisco fall well within the range of experimental error (Table IVA). This method of treating the data is especially useful in differentiating serotypes. Fraser and Berman (1965) consider that a SPD of less than 3 in their complement-fixation test indicates identity, whereas a difference greater than 3 indicates differentiation of serotype. In our hands, with the more precise micro-IF test, a SPD of more than 2 indicates differentiation of serotype, so that less than 2 may be taken to indicate identity.

Inter-relationships between serotypes are, however, better demonstrated by considering percentage cross-reactions (Wang and Grayston, 1970). These are shown in Table IIIB, which indicates that antisera to TRIC Type G show strong cross-reactions against both TRIC Type F and LGV Type III antigens (50 per cent.), whereas Type G antigens give only 25 per cent. cross-reactions with TRIC Type F and 13 per cent. with LGV Type III antisera. Thus the G serotype would appear to represent a connecting or an intermediate type between TRIC Type F and LGV Type III.

Wang and Grayston (1970) applied to TRIC and LGV serotypes the concept of 'senior' and 'junior' strains first suggested for influenza A viruses (Fazekas de St. Groth, 1969). This theory postulated that the antisera to senior strains cross-react to a higher degree than antisera to less dominant or junior strains. In the present instance, Type G strains would seem to be senior to the LGV III serotype. Senior or dominant strains are presumed by the theory to be newly evolving types, so that Type G could be regarded as being derived from LGV III and as forming a transitional form between this and TRIC serotypes.

The new serotype thus lies between the TRIC F and LGV III serotypes, but the micro-IF test cannot indicate whether G is a TRIC or an LGV agent. However, other biological properties make this clear.

Each of the isolates so far studied has come from the eye, genital tract, or rectum. Four of the TRIC Type G isolates came from one epidemiologically associated group of persons in London; namely IOL-202/ON from the eye of a baby with ophthalmia neonatorum, IOL-201/GCx from the cervix of the mother, IOL-238/R from her rectum, and IOL-200/GU from the father's urethra (Dunlop and others, 1973) Three further isolates came from another epidemiological group of persons, not known to have any association with the first group. These isolates were IOL-215/GU from a man who presented with urethritis, IOL-241/GCx from the cervix of one of his consorts; a second consort provided a further isolate IOL-240/CCx but this was not available for serotyping. The widespread distribution of TRIC Type G is suggested by the identification as Type G of the isolate Cal-392/OC, from the eye of a man suffering from conjunctivitis, that was kindly supplied from San Francisco by Dr J. Schachter (1971).

Thus all these isolates have come from genital, rectal, or genitally associated ocular disease, of the sort commonly seen with infection by TRIC serotypes D, E, or F. Although one isolate, IOL-238/R, came from the rectum, this case did not resemble those of LGV (Dunlop and others, 1973).

The patterns of growth in the yolk sac of the developing chick embryo and in irradiated McCoy cells, the sulphadiazine sensitivity as well as the pathogenicity for mice and baboons are all typical of Subgroup A *Chlamydia*. The characteristics of growth in the yolk sac and the lack of pathogenicity for mice by the intracerebral route, however, were more typical of TRIC agents than of LGV agents.

The pathogenicity of IOL-238/R for the baboon conjunctiva is within the range typically shown by other TRIC agent isolates (Wang and Grayston, 1971; Darougar and others, 1971). It is of interest that evidence of multiplication and infection by this agent has been demonstrated in the baboon urethra and that this was associated with histological evidence of inflammation of the urethra with lymphoid follicles in the mucosa. Kuo, Wang, and Grayston (1972) have recently shown that the growth of TRIC agent in HeLa 229 cell culture can be enhanced by pre-treatment of the cells with DEAE-Dextran. This enhancement of growth is not shared by LGV agents, so that this phenomenon would appear to provide a useful laboratory test to distinguish between TRIC and LGV isolates. The growth of IOL-238/R in HeLa 229 cells is enhanced by DEAE-Dextran, thus providing further evidence that serotype G is a TRIC rather than an LGV agent.

It is therefore proposed to refer to this newly defined serotype of *Chlamydia* as micro-IF Type G TRIC agent.

Summary

The existence of a newly defined Subgroup A *Chlamydia* serotype has been demonstrated by the micro-immunofluorescence typing test. This serotype would appear to lie between the LGV III and the TRIC F serotypes.

The seven isolates which fall into this new serotype have all been isolated from genital or rectal infection, or from genitally associated ocular disease of the sort commonly caused by TRIC Types D, E, and F.

The other biological characteristics of these isolates, including growth in yolk sac, cell culture enhancement of growth in HeLa cells pre-treated with DEAE-Dextran, pathogenicity for mice by intracerebral inoculation, and pathogenicity for the baboon conjunctiva, are similar to those of TRIC rather than LGV agents. Hence it would appear appropriate to refer to this new serotype as TRIC Type G.

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Caractérisation par immunofluorescence d'un nouveau sérotype de *Chlamydia:* TRIC Type G

SOMMAIRE

L'existence d'un nouveau sérotype du sous-groupe A a été démontré par l'épreuve de micro-immunofluorescence de typage. Le sérotype apparaîtrait comme se situant entre les sérotypes LGV III et TRIC F.

Les sept isolements qui se classent dans ce nouveau sérotype ont tous été isolés d'une infection génitale ou rectale ou d'une infection génitale associée à une atteinte oculaire comme ce que donne habituellement les TRIC de Type D, E et F. Les autres caractères biologiques de ces isolements, comprenant la culture sur membrane vitelline, l'accroissement de la pousse de la culture cellulaire sur cellules HeLa pré-traitées par DEAE-Dextrane, le pouvoir pathogène pour la souris après inoculation intra-cérébrale et ce pouvoir pour la conjonctive du babouin, ressemblent plutôt au TRIC qu'aux agents LGV. Il paraît donc approprié de mentionner ce nouveau sérotype en tant que TRIC de Type G.